14. The method of claim 6, wherein the mixture is exposed to the light for a period of 10 to 20 minutes.

REMARKS

Claims 6-10 are currently pending in the application. Claims 1-5 have been withdrawn from consideration. Claim 6 has been amended. New claims 11-14 have been added. Support for the amendment and the new claims may be found throughout the application as filed including, but not limited to: claims 6 and 10 as originally filed, page 5, lines 8-13; page 7, lines 3-13; page 15, lines 8-20; page 11, lines 14-18; and figure 3. The amendments add no new matter and are otherwise proper. In view of the following remarks, reconsideration and withdrawal of the rejections in the Office Action is respectfully requested.

1. Rejection of Claims 6-10 Under 35 U.S.C. § 112, First Paragraph

In the Office Action, the Examiner rejected all of the pending claims, claims 6-10, under 35 U.S.C. § 112, first paragraph, alleging that the specification is enabling only for certain types of cancer, but not cancer cells generally. Applicants respectfully traverse.

In support of the theory that the cancer therapy art is highly unpredictable, the Examiner cited Dyer, stating "the Dyer reference shows crystal violet to be inactive against spontaneous mammary carcinoma in vivo (No. 3513, page 123) the invention commensurate in scope with these claims." However, there are important differences between the treatment described by Dyer and the treatment described in the pending application. First, as discussed in greater detail below, the conventional chemotherapeutic methods referred to by Dyer are substantially different from the *photo*chemotherapeutic methods disclosed in the present invention. Therefore, the poor efficacy of crystal violet as a conventional chemotherapy agent against various cancers does not provide any direct information with regard to the efficacy of crystal violet as a *photo*chemotherapy agent. Second the treatment proposed by Dyer attempts to destroy tumors on the basis of the systemic toxicity of the chemotherapy agents. In this type of treatment, high quantities of the chemotherapy agent are taken over extended periods of time. At such high concentrations, selective uptake by

cancerous cells may be destroyed. Indeed, the fact that the crystal violet of Dyer did not perform well suggests that selective uptake was not achieved. As a result, the failure of the drug to treat the tumor may have been due to the development of severe side effects before the tumors could be treated. In photochemotherapeutic applications, much smaller doses of the crystal violet are required over shorter periods of time. This allows the crystal violet to be taken up preferentially by the cancerous cells and minimizes the potential side effects of toxicity, allowing the treatment to proceed until tumor growth is slowed, or the tumor is killed. For these reasons, Dyer does not support the conclusion that the methods of cancer treatment described in the pending application are unpredictable in nature.

The standard for enablement is whether one skilled in the art could make and use the invention in view of the teachings in the specification. Applicants submit that the Examiner in this case is improperly holding that the invention is enabled only for the disclosed working example. The Examiner lists the unpredictable nature of the invention, the state of the prior art, the lack of guidance and working examples, and the extreme breadth of the claims, as the basis for the assertion that one skilled in the art could not practice the entire scope of the claims without undue experimentation. Specifically, the Examiner asserts that because the cancer therapy art is highly unpredictable and no examples exist to demonstrate the efficacy of a triphenyl-methane dye with radiation against cancer cells generally, one skilled in the art could not practice the scope of the claimed invention without undue experimentation.

Applicants submit that in view of the nature of the invention and the disclosure of the specification, the knowledge available at the time of the invention, and the level of skill in the art at the time of the invention, one of skill in the art would not have to engage in undue experimentation to practice the invention embodied in the pending claims. Amended claim 6 recites a method for treating a mixture of cancerous and non-cancerous cells by: 1) contacting the mixture with a compound selected from a group of triarylmethane dyes which are preferentially taken up by the cancer cells; and 2) exposing the mixture to light that is capable of activating the triarylmethane dyes to selectively destroy the cancer cells. The invention is based on the inventor's surprising and unexpected discovery that these dyes have structural features that make them

particularly suitable for selective uptake by cancer cells. This uptake is driven, at least in part, by the enhanced mitochondrial membrane potential of the cells. This is discussed at length throughout the specification and is illustrated using leukemia cells as a representative example.

The mechanism for the selective uptake of the triarylmethane dyes is not unique to leukemia cancer cells. In fact, in light of the disclosure in the pending application, one of skill in the art would understand that the mechanism operates according to the same basic principles in most cancer cells. As noted above, the present invention operates on the principle that the triarylmethane dyes selectively accumulate in cancer cells to a greater extent than other presently known photochemical agents. This preferential accumulation has been shown to be controlled primarily by membrane potential-driven electrophoresis and has further been shown to track the uptake of Rh 123. In Mitochondrial Membrane Potential in Living Cells, Ann. Rev. Cell Biol., 4, 155-181 (1988), Chen reported the results of a comprehensive investigation of the selective uptake of Rh 123 by more than 200 cell lines/types. The results demonstrated that enhanced mitochondrial membrane potential is a prevalent cancer cell phenotype. In fact, of the cells studied, only 2% of the cells did not exhibit this phenotype. A copy of the Chen article is enclosed for the Examiner's convenience. Therefore, based on the disclosures of the Chen article and the pending application, one of skill in the art would understand that selective uptake of the triarylmethane dyes is applicable to cancer cells generally.

Moreover, the photochemical mechanism of phototoxicity is not unique to leukemia cancer cells. The photochemical mechanism of phototoxicity for TAM^+ dyes, such as crystal violet, is discussed in detail on pages 21 through 24 of the pending application. Briefly, this mechanism involves the interaction of light with a TAM^+ dye to produce reactive phototoxic species. Such photochemical activation mechanisms have been well known in the art for years and one of skill in the art would recognize that the mechanism is not unique to leukemia cancer cells. To the contrary, the mechanism of phototoxicity is broadly applicable to a wide variety of both cancerous and healthy cells. It is for precisely this reason that crystal violet and other

triarylmethane dyes, having a high selectivity for cancer cells, are so valuable in the field of photochemotherapy.

Thus, based on the discussion above, it seems clear that one skilled in the art would understand from the specification of the pending application that crystal violet and other triarylmethane dyes will preferentially accumulate in and kill a wide variety of cancer cells. Furthermore, conventional chemotherapeutic and photochemotherapeutic agents have been known and used for many years and one skilled in the art of administering such agents would be able to substitute the crystal violet taught by the pending application for those agents that were well known at the time the invention was made without undue experimentation.

However, the Examiner need not rely only on the evidence outlined above. Enclosed is the affidavit of Dr. Guilherme L. Indig which presents data showing the efficacy of crystal violet as a photochemotherapeutic agent against human adenocarcinoma cells and human uterine sarcoma cells. Both of these are hard tumors which differ markedly from the leukemia cells described in the pending application. The studies of the human adenocarcinoma and human uterine sarcoma cells were obtained using substantially the same procedures as described on pages 9-11, 14, and 15 of the pending application. These data firmly establish that the methodology described and claimed in the present invention may be applied generally and without substantial modification or experimentation to a broad range of cancerous cells using the triarylmethane dyes recited in claim 6.

Therefore, based on the nature of the invention, the knowledge available at the time of the invention, and the level of skill in the art at the time of the invention, one of skill in the art would not have to engage in undue experimentation to practice the invention embodied in the pending claims. For this reason, Applicants respectfully request that the Examiner withdraw the rejection.

2. Rejection of Claims 6-10 Under 35 U.S.C. § 112, Second Paragraph

In the Office Action, the Examiner rejected claims 6-10 as being indefinite because the claims fail to recite the amount of the triarylmethane dye being used. Applicants have amended claim 6 to clarify that the mixture of cancerous and non-

cancerous cells is exposed to an "effective amount" of the triarylmethane dye, that is, an amount that is effective to selectively kill or inhibit the growth of cancer cells in the mixture. Applicants note that the term "effective amount" is not indefinite provided one skilled in the art could determine specific values for the amount based on the disclosure. (MPEP 2173.05(c)) The pending application provides sufficient guidance to allow one of skill in the art to determine what is meant by an "effective amount." The discussion on page 14, line 20 through page 15, line 20 provides a working example that includes a specific value of an effective amount of crystal violet in a suspension of leukemia cells and healthy murine CFU-GM cells. Thus, Applicants believe that the amendment to claim 6 renders that claim definite. Therefore, Applicants respectfully request that this rejection be withdrawn.

3. Rejection of Claims 6-10 Under 35 U.S.C. § 103(a), First Paragraph

In the Office Action, the Examiner rejected each of the pending claims, claim 6-10, under 35 U.S.C. § 103(a) alleging that the claims are unpatentably obvious over Dyer in view of the Manual of Oncology Therapeutics, authored by Kay See-Lasley (hereinafter "See-Lasley"). Specifically, the Examiner stated that it would be obvious to one of skill in the art to combine Dyer, which teaches crystal violet as an effective agent against carcinomas and sarcomas by intratumoral administration, with See-Lasley, which teaches a combination therapy involving both conventional radiation therapy and chemotherapy. The Examiner further stated that the results obtained by such a combination would be "no more than the additive effects of the ingredients." However, the Examiner did concede that a showing of greater than additive effects attained by the combination would overcome this rejection.

The Examiner has failed to establish a prima facie case of obviousness. In order to establish a prima facie case of obviousness, the prior art references alone or in combination must teach each and every limitation of the rejected claims. Even in combination, the prior art cited by the Examiner fails to teach each and every limitation of rejected claims 6-10. Specifically, the prior art relied upon by the Examiner does not teach a photochemical method for treating cancer which includes the steps of contacting the cancer cell with a photochemical agent and radiating those cells *with*

light of a wavelength suitable for photoactivate the photochemical agent as recited in claim 6 of the pending application.

The combination of Dyer and See-Lasley teaches only a method wherein cancer cells are contacted with a conventional chemotherapeutic agent and the same cells are exposed to ionizing electromagnetic radiation having a wavelength suitable to destroy the cancer cells. More particularly, Dyer teaches that crystal violet may be used as a chemotherapy agent to treat carcinomas and sarcomas in humans using intratumoral administration. Chemotherapy is a term that covers a class of cancer treatments that operate by treating a disease by means of chemicals that have a toxic effect upon or that selectively destroy cancerous tissue. (*See, e.g.,* the online medical dictionary at http:\\cancerweb.ncl.ac.uk\omd\ A copy of the relevant section of this webpage is enclosed for the Examiner's convenience.) As noted by the Examiner, this reference does not teach the irradiation of the crystal violet. See-Lasley teaches using a standard combination of radiation therapy and chemotherapy to treat cancer cells. Radiation therapy is a method of treating cancer cells by irradiating them with ionizing electromagnetic radiation such as X-rays or gamma rays. In radiation therapy, it is the high-energy radiation itself that destroys cancerous tissues. (*See, e.g.,* Harrison's Principles of Internal Medicine, 13th ed., p. 1828.) The energy of the radiation used in conventional radiation therapy is too high to photoactivate the triarylmethane dyes and produce phototoxic effects. Thus, See-Lasley actually teaches two separate therapies, chemotherapy which operates through a chemical reaction between a cancerous cell and a chemotherapeutic compound and radiation therapy which kills cancer cells through the direct interaction of high-energy radiation with the cells. Thus, neither cited reference teaches exposing cells to light having a wavelength suitable to photoactivate the crystal violet or another triarylmethane dye, resulting in the selective destruction of cancer cells.

In contrast to the cited references, the present invention provides a method for treating cancer cells wherein drug efficacy results from the interaction between the light and the photochemical compound, which serves to activate the compound and produce phototoxic effects on cancerous cells. As such, the result is much more than just the additive effects of the two ingredients, rather it is the unique result of a

synergistic relationship between the triarylmethane dye and the radiation. The Examiner's suggestion that the results of the present invention are no more than the additive effects of the separate ingredients is inaccurate. As discussed in the specification on page 10, line 23 through page 11, line 7, crystal violet has negligible toxicity toward cancerous cells in the absence of radiation having a wavelength sufficient to photoactivate the crystal violet. Similarly, radiation having a wavelength sufficient to photoactivate the triarylmethane dyes has negligible toxicity toward cancerous cells in the absence of the dyes. Therefore, if the methods recited in claims 6-10 of the pending application provided no more than the additive effects of the two ingredients, the methods would produce a negligible toxicity toward cancerous cells. This is simply not the case. As amply demonstrated by the example and discussion of the pending application, the claimed methods demonstrate a pronounced and highly selective phototoxicity toward cancerous cells.

Moreover, even if the cited references did teach the use of crystal violet as a phototoxic agent, the references would not render claims 6-10 of the pending application obvious. As noted above, the present invention is based on the inventor's surprising and unexpected discovery that certain triarylmethane dyes, such as crystal violet, have certain structural features that enable them to selectively destroy cancer cells without substantially destroying non-cancerous cells. Although it has been recognized in the past that triarylmethane dyes might have the capability to destroy cells through a phototoxic mechanism (see, for example, Indig *et al.*, Recent Res. Devel. In Pure & Applied Chem., Vol. 3, pages 9-19 (1999)) , it was not previously known that crystal violet or other triarylmethane dyes could be administered in a manner that would result in the preferential uptake of the crystal violet by cancer cells and the selective destruction of cancer cells relative to non-cancerous cells. The references cited by the Examiner do not teach or suggest a photodynamic therapy based on the selective destruction of cancer cells. Therefore, the claims of the present invention are patentable over the cited references.

For each of the reasons discussed above, the invention is not obvious in light of the cited art and Applicants respectfully request that the Examiner withdraw this rejection.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants respectfully request that the Examiner reconsider and withdraw the rejections discussed above. If Examiner Goldberg has any questions or believes a telephone discussion would expedite prosecution, he is invited to contact the undersigned.

Respectfully submitted,

Date: September 24, 2002

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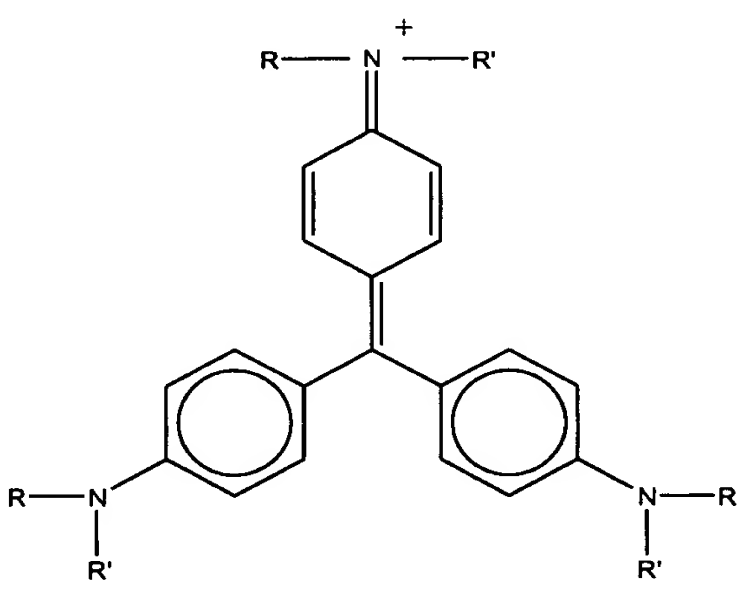
CLAIM AMENDMENTS

6. A method of selectively killing cancer cells or inhibiting growth of cancer cells in a mixture of cancerous and non-cancerous cells[*in vitro, in vivo, or ex vivo*], the method comprising:

(a) contacting the mixture of cancerous and non-cancerous [cancer] cells with an effective amount of a compound having the structure [selected from the group consisting of]:



wherein each R and R′ is independently selected from the group consisting of hydrogen and methyl groups [C_1-C_6 linear or branched alkyl], and further wherein the compound exhibits preferential uptake by the cancerous cells compared with the non-cancerous cells; and

(b) exposing the mixture of cancerous and non-cancerous [cancer] cells from [step] (a) to light [radiation] of a suitable wavelength to photoactivate the compound, wherein the compound exhibits selective phototoxicity toward the cancerous cells over the non-cancerous cells [whereby cancer cell death or cancer growth inhibition results].

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MITOCHONDRIAL MEMBRANE POTENTIAL IN LIVING CELLS

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CONTENTS

INTRODUCTION 155
RHODAMINE 123 AS A UNIQUE PROBE FOR MITOCHONDRIAL MEMBRANE POTENTIAL 157
MONITORING MITOCHONDRIAL MEMBRANE POTENTIAL 161
In a Single Cell 161
In Heterokaryons 162
In Relation to Cell Growth 164
In Relation to Cell Differentiation 164
In Relation to Cell Motility 165
EFFECTS OF VARIOUS AGENTS ON MITOCHONDRIAL MEMBRANE POTENTIAL 165
MITOCHONDRIAL MEMBRANE POTENTIAL IN CANCER CELLS 167
EXPLOITATION OF MITOCHONDRIAL MEMBRANE POTENTIAL 168
As a Marker for Localization 168
As a Marker for Flow Cytometry 172
As a Means for Accumulating Drugs 172
CONCLUDING REMARKS 175

INTRODUCTION

A proton gradient exists across the mitochondrial inner membrane as a result of proton pumping by the respiratory chains located in this membrane (Mitchell 1966; Boyer et al 1977; Tzagoloff 1982; Lane et al 1986; Murphy & Brand 1987). This gradient has two components: a membrane potential (protons are positively charged) and a pH gradient (protons also determine acidity). The energy stored in either the pH gradient or the membrane potential drives the synthesis of ATP by F_0F_1ATPase, which functions as a proton turbine (Mitchell 1979; Tzagoloff 1982; Racker 1985; Boyer 1987, 1988; Pedersen & Carafoli 1987; Pedersen et al 1987). It

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is believed that mammalian mitochondria express this electrochemical gradient mostly as membrane potential at around 180 mV and to a smaller extent as pH gradient at approximately one unit (equivalent to 60 mV), which yields a total energy of approximately 240 mV (Skulachev & Hinkle 1981). The magnitude of the proton gradient in different cell types and the ratio of membrane potential to pH gradient chosen for its expression in living cells are, however, poorly understood (Tzagoloff 1982). For example, during cell differentiation energy requirements may change. When myoblasts fuse to form myotubes, the magnitude of the mitochondrial proton gradient and the ratio of its components may change to meet the need of contraction; but at present we do not know whether the state of the proton gradient across the inner mitochondrial membrane is the same in myoblasts and myotubes. As formulated by Mitchell, theoretically the proton gradient may be expressed either mostly as membrane potential, or mostly as pH gradient, or partly as membrane potential and partly as pH gradient. Conceivably, certain cell types might express it mostly as pH gradient or membrane potential in order to meet some special needs of their differentiated functions or as a result of an aberrant state such as cancer. Regulation of the proton gradient could play a fundamental role in cellular programs since each of its two components affects the transport of metabolites and ions, including calcium (LaNoue & Schoolwerth 1979). The membrane potential also influences the import of precursor enzymes (Schleyer et al 1982; Verner & Schatz 1987; Chen & Douglas 1987; Horwich et al 1987; Eilers & Schatz 1988) and mitochondrial protein synthesis (Rabinowitz et al 1977; Abou-Khalil et al 1986).

We do not know how closely isolated mitochondria in vitro mimic the proton gradient of mitochondria within living cells. Mitochondria are constantly interacting with cytoplasm, and very little is known about the nonsubstrate factors that influence mitochondrial respiration, as well as proton gradients, in vivo. If we were to compare mitochondrial proton gradients before and after myoblast fusion, it would be more sensible to study them directly in living cells. Determining the proton gradient across mitochondria in a single living cell (as opposed to using a Clark electrode to measure the average rate of respiration in ten million cells) seems essential for such studies.

Approaches involving fluorescence may be logical in view of their noninvasive nature, the existence of more than a hundred thousand fluorescent dyes, the development of a highly sensitive detection system (thanks to astronomy), and changes in fluorescent characteristics in response to changes in the environment. Indeed, monitoring NADH fluorescence for assessing mitochondrial bioenergetics in living cells was pioneered many years ago by Chance (1970). NADH is a useful indicator of the oxygen

requirements of energy-linked functions (Chance 1976); this approach has been used to monitor NADH-linked bioenergetics in tumor, liver, heart, brain, skeletal muscle, sperm, and other cells and tissues. Extrinsic fluorescent dyes, such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and oxonol V, that reflect the energized state of isolated mitochondria in vitro have also been developed (see review by Bashford & Smith 1979). These pioneering works have provided the necessary groundwork for further research.

Lipophilic compounds with a positive charge delocalized throughout the whole molecule equilibrate according to the Nernst potential (Rottenberg 1979; Lichtshtein et al 1979; Deutsch et al 1979; Freedman & Laris 1981; Brand & Felber 1984; Ritchie 1984). By quantitating the mitochondrial uptake of lipophilic cations in the absence of a plasma membrane potential, one might be able to monitor mitochondrial membrane potential (Lichtshtein et al 1979; Ritchie 1984; Davis et al 1985). Lipophilic cations such as tetraphenylphosphonium (TPP) have long been used to probe the membrane potential of isolated mitochondria in vitro and of bacteria (Grinius et al 1970). Fluorescent lipophilic cations such as cyanines have been used to measure the plasma membrane potential in living cells for many years (Sims et al 1974; Hoffman & Laris 1974; Laris et al 1975, 1976; Waggoner 1976, 1979; Tsien & Hladky 1978; Philo & Eddy 1978; Cohen & Salzberg 1978; Shapiro et al 1979; Freedman & Hoffman 1979; Bashford & Smith 1979; Rink et al 1980; Cohen et al 1981; Freedman & Laris 1981; Johnstone et al 1982). Cyanines and another fluorescent lipophilic cation, safranine, have also been used to monitor mitochondrial membrane potential (Colonna et al 1973; Akerman & Wikstrom 1976; Akerman 1979; Akerman & Jarvisalo 1980; Tatham & Delves 1984; Wilson et al 1985).

RHODAMINE 123 AS A UNIQUE PROBE FOR MITOCHONDRIAL MEMBRANE POTENTIAL

Rhodamine 123 (Rh123; Figure 1) is a highly specific fluorescent dye for mitochondria in living cells (Figure 2). Its discovery resulted from a

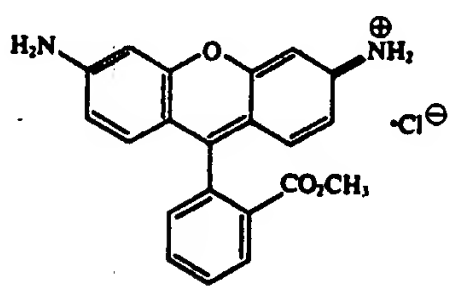

Figure 1 Structure of rhodamine 123.

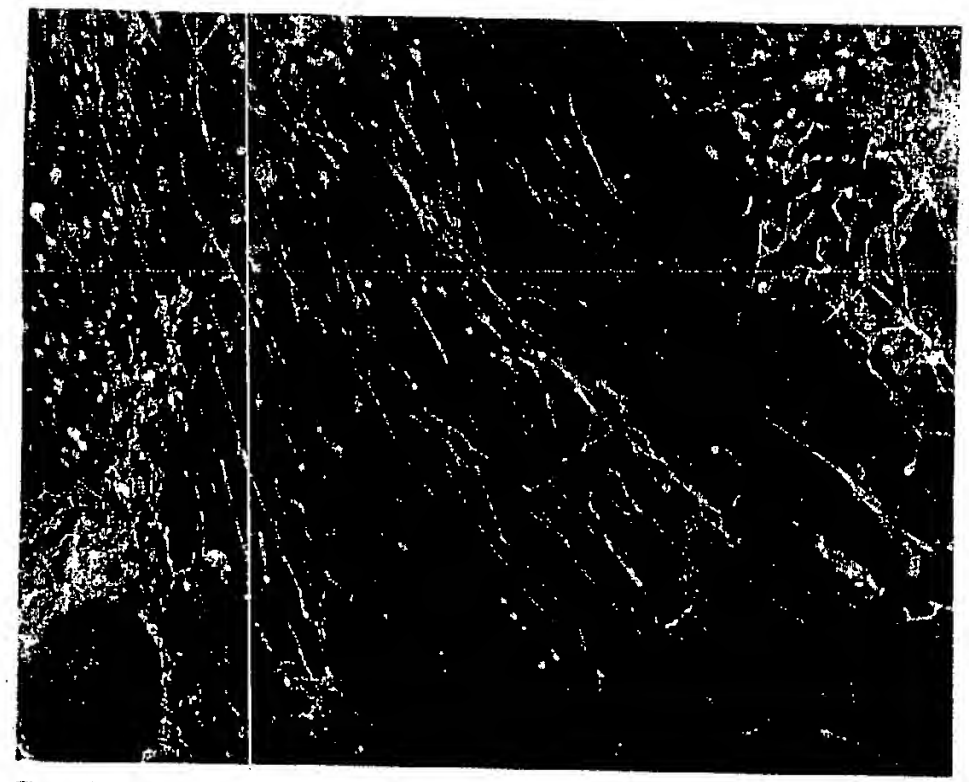

Figure 2 Mitochondria in living human fibroblasts stained with rhodamine 123 (10 μg/ml for 10 min at 37°C). Magnification 1500×.

serendipitous observation (Walsh et al 1979; Johnson et al 1980; Chen et al 1982). It is a fluorescent lipophilic compound with a delocalized positive charge ($pK_a > 10$). It has an excitation spectrum with the maximum of 500 nm and a molar extinction coefficient of $7.5 \times 10^4\ cm^{-1}\ mol^{-1}$ in water (Darzynkiewicz et al 1981). The absorption spectrum does not change with dye concentrations. However, the peak of its emission spectrum varies in response to concentrations, for example at 10^{-8} M, the maximum is about 525 nm and at 10^{-3} M, 545 nm, which agrees with a typical red shift. Using computer simulation Darzynkiewicz et al (1982) showed that the concentration-dependent red shift of the emission results from an inner filter shift effect, not from excimer formation. In contrast to other rhodamine dyes, which can only be excited by green light to yield red fluorescence, Rh123 can be excited by blue light (485 nm) to green light (546 nm) and emits yellowish green to red fluorescence. The emission spectrum of the Rh123 in mitochondria has a red shift of 12 nm when compared with that in water (Darzynkiewicz et al 1982; Emaus et al 1986). This shift is probably due to the formation of chemical complexes, not to an inner

filter effect or excimer generation. For microscopy and flow cytometry, the optimal conditions is to use blue excitation in order to yield greenish fluorescence and the filter set normally used for fluorescein dyes, not rhodamine dyes.

That mitochondrial membrane potential is indeed the driving force for Rh123 uptake has been tested in numerous experiments (Johnson et al 1981; Maro et al 1982; Davis et al 1985; Hollenbeck et al 1985; Emaus et al 1986; Modica-Napolitano & Aprille 1987). Ionophores that dissipate the mitochondrial membrane potential, such as valinomycin (for potassium ions), *p*-trifluoromethoxyphenylhydrazone (FCCP, for protons), and dinitrophenol (DNP, for protons), prevent Rh123 uptake. Electron transport inhibitors such as azide, antimycin A, or rotenone reduce uptake in cells that do not have a high rate of glycolysis, and in conjunction with oligomycin, completely eliminate Rh123 uptake by all cells. These conditions prevent the generation of a membrane potential through reverse F_0F_1ATPase powered by ATP from glycolysis. Anaerobic conditions also reduce Rh123 uptake as expected. Inhibitors of mitochondrial protein synthesis (chloroamphenicol), cellular protein synthesis (cycloheximide), and RNA synthesis (actinomycin D) have no effect on uptake. The most compelling evidence comes from a nigericin experiment. During continuous respiration nigericin hyperpolarizes mitochondria by an electrically neutral exchange of protons and potassium ions, which decreases the pH gradient and increases the mitochondrial membrane potential (Reed 1979). Indeed, nigericin dramatically increases Rh123 uptake in cell types that have a low mitochondrial membrane potential and a high pH gradient, including normal African green monkey kidney epithelial cell line, CV-1, and fes oncogene-transformed mink fibroblasts (Johnson et al 1981, 1982; Davis et al 1985). Using isolated mitochondria, Emaus et al (1986) and Modica-Napolitano & Aprille (1987) also demonstrated a direct correlation between mitochondrial membrane potential and Rh123 uptake (Figure 3). Thus, Rh123 uptake is most likely driven by the mitochondrial membrane potential.

Because mitochondria are enclosed within cells, Rh123 uptake by mitochondria in living cells should also be affected by the plasma membrane potential. The latter may concentrate Rh123 in the cytoplasm relative to the medium because it is also inside negative. Indeed, in the presence of 137 mM K^+, which dissipates the plasma membrane potential, mitochondrial uptake of Rh123 is reduced (Davis et al 1985). Since a 137-mM K^+ medium does not have deleterious effects on short term experiments, mitochondrial membrane potential can be monitored in this medium without the influence of the plasma membrane potential (Davis et al 1985). Moreover, in conjunction with nigericin, the proton gradient (membrane potential and pH

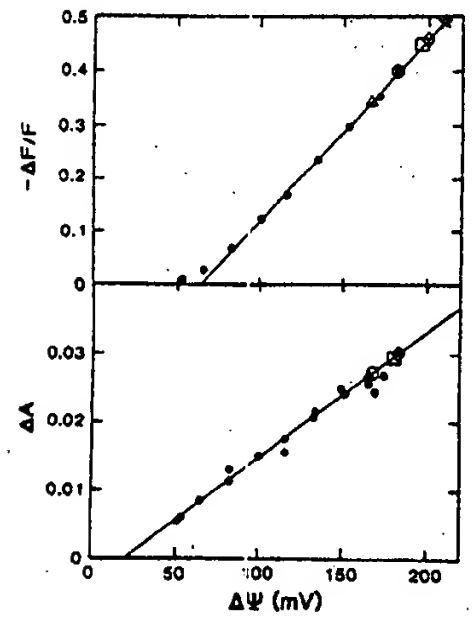

Figure 3 Rhodamine 123 uptake by isolated mitochondria in vitro as a function of membrane potential. Rhodamine 123 spectral changes and potassium diffusion potentials in rat-liver mitochondria. Rhodamine 123 absorbance and fluorescence signals were extrapolated backwards to the time of valinomycin addition. ΔA and ΔF are differences between extrapolated absorbance and fluorescence values and values obtained after carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) treatment. F is fluorescence after CCCP. $\Delta\psi$ was varied by varying KCl between 0.1 and 16 mM and mannitol between 39.8 mM and 8 mM. Open symbols show where mitochondria incubated under various metabolic conditions fall on the calibration curve: □, plus succinate (State 4); △, plus succinate and ADP (State 3); ○, plus succinate without added P_i (closed circles are the result of superimposed data); ◇, plus 5 mM glutamate and 5 mM malate (State 4) (from Emaus et al 1986).

gradient) can also be monitored (Johnson et al 1982; Davis et al 1985). The difference in dye uptake before and after nigericin may be indicative of the magnitude of the pH gradient. (Because nigericin induces hyperpolarization of the plasma membrane potential, it is necessary to include ouabain in the medium.)

There are numerous fluorescent, lipophilic, delocalized cations synthesized by the photographic industry. They should, in principle, all be useful for probing mitochondrial membrane potential. Is Rh123 unique? Initially, rhodamine 3B, 6G, safranine, and numerous cyanines were all found suitable for mitochondrial localization (Johnson et al 1980, 1981); it was then realized that most lipophilic cations are quite toxic to cells (Johnson et al 1982; Lampidis et al 1982, 1983, 1984, 1985). Tests of more than 3000 fluorescent lipophilic cations in the course of 9 years has indicated that Rh123 is the least toxic (own unpublished results). Remarkably, cells like CV-1 are able to grow normally in Rh123 at 10 μg/ml for two weeks (Lampidis et al 1983). Although Rh123 was later found to be toxic to certain carcinoma cells under certain conditions (see below), the procedure used for staining mitochondria in living cells, 10 μg/ml for 10 min at 37°C, remains relatively nontoxic to all cell types so far tested. This conclusion is drawn from numerous experiments performed during the past 9 years in various laboratories (Johnson et al 1980, 1981,

1982; Chen et al 1982, 1983, 1984, 1985b; James & Bohman 1981; Albertini 1984; Gundersen et al 1982; Arslan et al 1984; Collins & Foster 1983; Ronot et al 1986; DeMartinis et al 1987). At 10 μg/ml for 10 min, Rh123 does not affect cell growth, DNA synthesis, RNA synthesis, protein synthesis, glucose transport, organization of endoplasmic reticulum and Golgi apparatus, expression of fibronectin, organization of microfilaments, microtubules and intermediate filaments, cell locomotion monitored by time-lapse videomicroscopy, and mitochondrial cristae and overall mitochondrial morphology examined by thin-section electron microscopy. However, prolonged exposure of cells to Rh123 is cytotoxic, especially to the carcinoma cells (see below).

In addition to low toxicity, there is another characteristic that makes Rh123 unique. Rh123 is the only fluorescent lipophilic cation tested thus far whose uptake by mitochondria in living cells can be completely eliminated by azide plus oligomycin. For most cell types, the other fluorescent dyes tested can still stain mitochondria significantly in the presence of azide plus oligomycin, apparently as a result of partitioning in membrane lipids (Terasaki et al 1984; Chen et al 1988). It is still unknown why other dyes, but not Rh123, can stain mitochondria in living cells in the absence of a mitochondrial membrane potential. Two possibilities are worth considering. Certain lipids of the mitochondrial inner membrane may bind other lipophilic cations, but not Rh123, in the absence of a membrane potential. For example, cardiolipin and phosphatidylglycerol, which are enriched in the mitochondrial inner membrane, may have a high affinity for rhodamines 3B and 6G and various cyanines, but not for Rh123. The other explanation is simple lipid partitioning. Rh123 is the least hydrophobic lipophilic cation tested. It is soluble in water at greater than 1 mg/ml. Between water and isopentane, Rh123 has a partition coefficient of 0.9 (favoring water); in contrast, 3,3′-dihexyloxacarbocyanine has a partition coefficient of 0.1 (favoring isopentane). Perhaps, in the absence of a mitochondrial membrane potential, other lipophilic cations have greater propensity to be partitioned into the inner membrane of mitochondria where the lipid density is the highest because of the infolding cristae (Terasaki et al 1984).

MONITORING MITOCHONDRIAL MEMBRANE POTENTIAL

In a Single Cell

Mitochondria in a given cell always have identical fluorescence intensities when exposed to Rh123 (Johnson 1980, 1981). Thus, it is likely that all mitochondria within a cell have an identical mitochondrial membrane

potential (Chen et al 1982, 1984, 1985b). This observation suggests that despite variations in size, shape, location, and distance among them, mitochondria respond to the same regulators by setting an equal electric potential. Whether the regulators are ADP, NADH, other substrates, oxygen, Ca^{2+}, certain macromolecules, or a combination of such agents is still unknown. Mitochondrial membrane potential in living cells is best monitored by quantitation of lipophilic cations in the presence of 137 mM K^+ in the medium (Figure 4; Davis et al 1985). Since the plasms membrane potential of a given cell type changes infrequently, the heterogeneity of mitochondrial membrane potential of a cell population of the same type can be monitored without using the high K^+ medium. Qualitative differences, which are often useful, can be documented by photography or videorecording. Flow cytometry with a photon-counting device has also become increasingly useful (Bertoncello et al 1985; Benel et al 1986; Doolittle et al 1987; Mulder & Visser 1987).

Rhodamine 123 has been used to assess the relative membrane potentials of the mitochondria in numerous normal cell types. The highest membrane potential was consistently found for the mitochondria of cardiac muscle cells, followed by (in order) those of skeletal muscle cells, smooth muscle cells, macrophages, hepatocytes, fibroblasts, resting neuronal cells, glial cells, and keratinocytes. The lowest values were found in bladder epithelial cells and resting T and B lymphocytes (Lampidis et al 1982; Summerhayes et al 1982; Chen et al 1982; own unpublished results). Nigericin fails to increase mitochondrial membrane potential in cardiac muscle cells, which indicates that the entire proton gradient is expressed as membrane potential in these cells. In some cell types, such as monkey kidney epithelial CV-1 cells and fes-transformed mink fibroblasts, the mitochondrial membrane potential is very low but increases dramatically upon treatment with nigericin and oubain, which suggests that a significant portion of the overall gradient is expressed as pH gradient in these cells (Johnson et al 1982; Davis et al 1985; Modica-Napolitano & Aprille 1987). The existence of cell types with such mitochondria poses an interesting question for the debate whether the proton gradient reaches the bulk water phase of the mitochondrial matrix. If this were the case, the pH in the mitochondrial matrix of CV-1 cells might be too high for the activity of many of the enzymes located there. Such a pH seems physiologically intolerable; hence, the regulation of the pH of the mitochondrial matrix is most likely dissociated from the proton circuit of the proton pump and proton turbine.

In Heterokaryons

In heterokaryons obtained by fusing cells with high mitochondrial membrane potentials such as MCF-7 (human breast carcinoma) with cells with low mitochondrial membrane potentials such as PtK2 (normal kangaroo-

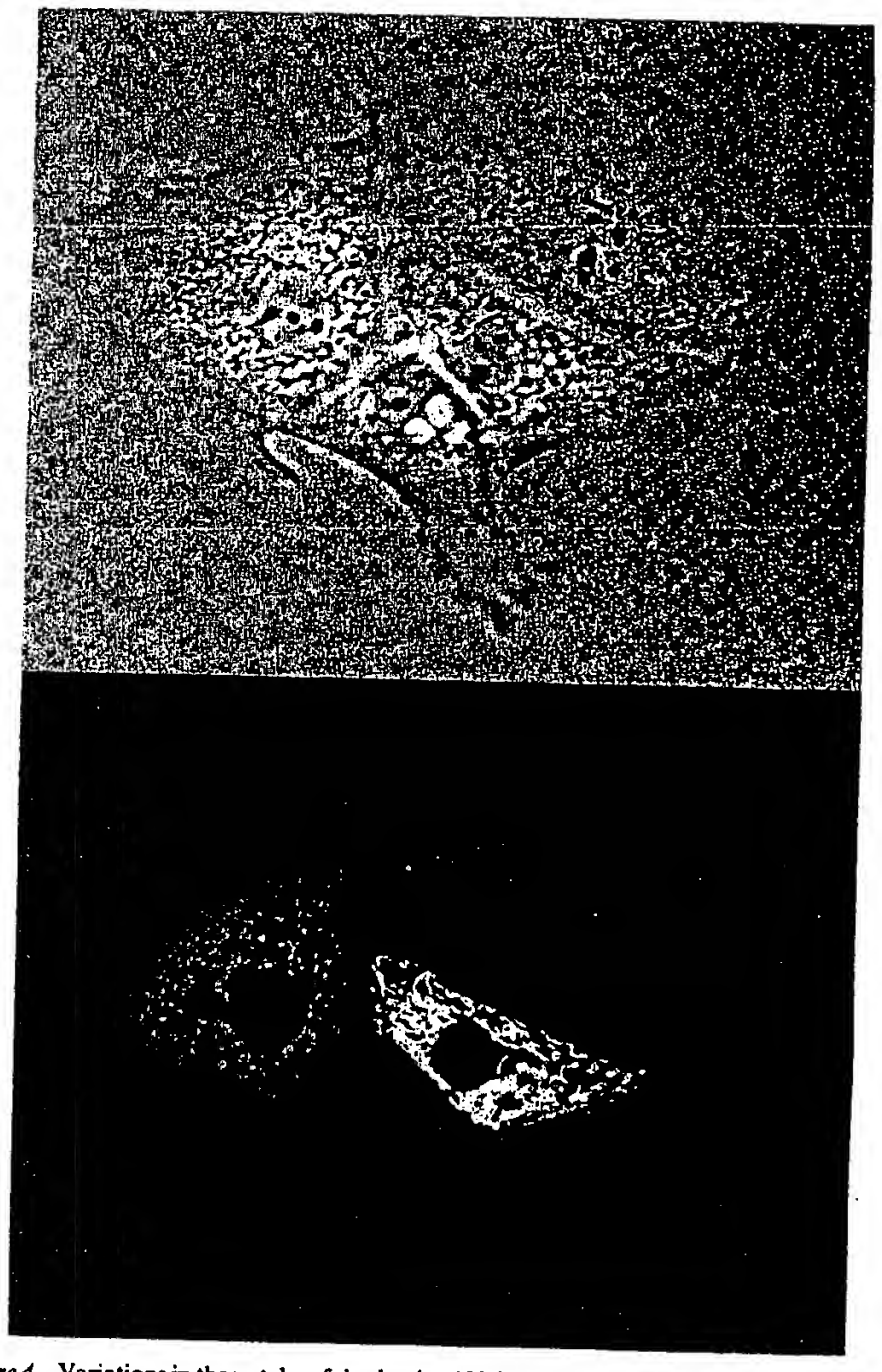

Figure 4 Variations in the uptake of rhodamine 123 (0.1 μg/ml for 3 hr in the presence of 137 mM K^+) by human embryonic kidney epithelial cells. Four different levels of mitochondrial membrane potential may be discerned. (*top*) phase-contrast; (*bottom*) epifluorescence. Magnification 730×.

rat kidney epithelium), differential uptake of Rh123 by the two different mitochondrial populations is detected immediately after fusion and is maintained for up to 6 hr (Chung et al 1988). The more diffusible molecules of the cytoplasm are expected to equilibrate at this time point, but not necessarily the macromolecules (such as cytoskeletal components) of the two cells. When a <6 hr old heterokaryon is processed for immunofluorescence staining with keratin antibodies, two distinct patterns of keratin, one from MCF-7 and the other PtK2, are observed. However, 18 hr after fusion, two distinct keratin patterns are no longer detectable, and all the mitochondria of the heterokaryon take up an equal amount of Rh123. Intriguingly, the level of uptake by these mitochondria is between that of unfused MCF-7 and PtK2. These observations suggest that mitochondrial membrane potential in living cells is influenced by unknown, slowly diffusible factors.

In Relation to Cell Growth

Darzynkiewicz et al (1982) reported that cultured L1210 mouse leukemic cells, Chinese hamster ovary cell line (CHO), and Friend erythroleukemic cells in stationary growth phase take up 30 to 45% less Rh123 than do cells growing exponentially. Changes in Rh123 uptake during the activation of human lymphocytes from a resting state to a growing state have also been described (Darzynkiewicz et al 1981). About 80% of lymphocytes stimulated by phytohemagglutinin (PHA) enter a new cell cycle, are found in the S, G2, or M phase of the cycle, and have a 5- to 15-fold increase in the Rh123 uptake (Darzynkiewicz et al 1981). Similar results were reported for hemopoietic stem cells (Bertoncello et al 1985), chondrocytes (Benel et al 1986), nonparenchymal liver cells (Doolittle et al 1987), and bone marrow cells (Mulder & Visser 1987). Goldstein & Korczack (1981) also reported that Rh123 uptake by human fibroblasts from the skin of a healthy donor, as well as a patient with Hutchinson-Gilford (progeria) syndrome (Goldstein 1978) is higher during their exponential growth than during their confluent resting state. Concomitant with a decrease in Rh123 uptake, there is a decrease in oxygen consumption. Moreover, at the mid-log phase, Rh123 uptake is higher in young fibroblasts from the healthy donor than in fibroblasts from the progeria subject. However, at the confluent stationary phase, these fibroblasts show no difference in Rh123 uptake.

In Relation to Cell Differentiation

When cells are induced to differentiate, mitochondrial membrane potential may change. Once myoblasts fuse into a myotube, the uptake of Rh123 at equilibrium is increased tenfold, which is equivalent to an increase of 60

mV in the proton potential of their mitochondria (Summerhayes et al 1982; Chen et al 1984, 1988). This observation suggests that soon after fusion, myogenesis involves a reprogramming of mitochondria to prepare for a higher rate of ATP consumption by contraction. In contrast, a decrease of 50–75% in Rh123 uptake was observed when HL-60 cells (human promyelocytic leukemia cells) differentiate into granulocytelike cells, or when Friend erythroleukemia cells differentiate into erythroidlike cells (James & Bohman 1981; Darzynkiewicz et al 1982; Collins & Foster 1983). Similarly, using a cyanine dye Levenson et al (1982) observed a reduction in mitochondrial membrane potential when Friend erythroleukemia cells were induced to differentiate by dimethyl sulfoxide. While the reduction in mitochondrial membrane potential when cells differentiate into erythroidlike cells may be rationalized by the fact that erythrocytes use glycolysis exclusively to generate ATP, it is difficult to explain the decrease observed in HL-60 cells unless one assumes that these differenting cells have also entered the pathway of cell death. Mature granulocytes should have active mitochondria, whereas dying cells invariably turn off respiration. Because of these findings, one may argue that HL-60 cells may not be a suitable system for studying alterations in mitochondrial membrane potential in the course of granulocytic differentiation.

In Relation to Cell Motility

The possible relationship between mitochondrial membrane potential and cell motility has been explored in mouse bladder epithelial cells in culture. In such a colony, cells at the leading edge always have a higher mitochondrial membrane potential than those located elsewhere (Johnson et al 1981). When a wound is made in confluent culture, cells along the edge of the wound increase mitochondrial membrane potential within a few minutes (Johnson et al 1981). These cells have active ruffling and displacement activities. Once the wound is healed and a confluent epithelium restored, the locomotive activities stop, and the mitochondrial membrane potential returns to the resting level. It is possible that migrating cells need more ATP to sustain locomotion, and an increase in mitochondrial membrane potential is indicative of a higher rate of mitochondrial ATP synthesis.

EFFECTS OF VARIOUS AGENTS ON MITOCHONDRIAL MEMBRANE POTENTIAL

Lampidis et al (1984) and Tapiero et al (1986) reported that the uptake of Rh123 by some tumor cells is increased by the calcium channel blocker

verapamil. Davis & Chen (1988) showed that in CV-1 and 64F3 (v-fes-transformed mink fibroblasts), which have low mitochondrial membrane potentials, the uptake of Rh123 or that of another lipophilic cation, tetraphenylphosphonium (TPP) is increased more than tenfold by verapamil, which corresponds to a potential increase of 60 mV. However, verapamil has no effect on a human breast carcinoma line, MCF-7, and a normal mink fibroblast line, CCL64, which already have high mitochondrial membrane potentials. Because the verapamil-induced increase in Rh123 or TPP uptake is unaffected by ouabain, it is unlikely that the plasma membrane is involved in the effects mentioned above. Azide or oligomycin alone only slightly inhibits the effect of verapamil on Rh123 or TPP uptake. When combined, they completely eliminate the hyperpolarization induced by verapamil. Nifedipine and diltiazem, other calcium channel blockers, also led to the hyperpolarization of mitochondria in cells with a low mitochondrial membrane potential.

The reduction in Rh123 uptake following treatment of mouse leukemic cells with anticancer drugs has been reported (Bernal et al 1983). It is irreversible and correlates well with the loss of clonogenic ability. Similar results were reported with cytotoxic nucleoside analogs on human lymphocytes (Verhoef et al 1986). Adriamycin first converts mitochondria from filamentous to granular forms; reduction in Rh123 uptake follows (Lampidis et al 1982; unpublished results). Methylglyoxal guanylhydrazone (MGBG) causes marked mitochondrial swelling, but no change in Rh123 uptake (Fleshkewych et al 1980; Kramer et al 1983; Nass 1984). Novobiocin, an inhibitor for topoisomerases, has a marked effect on mitochondrial morphology, but not on Rh123 uptake (Downes et al 1985).

The mechanism for the antispermatogenic effect of gossypol, a drug widely used in China, is still unknown (Chang et al 1980); mitochondria have been suggested as a potential target (Hoffer 1982; Wichmann et al 1983). Tanphaichtr et al (1984) and Robinson et al (1986) reported that gossypol reduces Rh123 uptake in a rat Sertoli-like cell line (TR-ST), advanced spermatids, and spermatozoa, but not in nontesticular somatic cell lines. Therefore, gossypol may selectively affect mitochondrial membrane potential in spermatogenic cells, but not in other cell types. Since the motility of sperm depends on ATP, a reduction in Rh123 uptake induced by gossypol may indicate reductions in mitochondrial membrane potential and ATP supply, which in turn may impair sperm motility as well as fertility.

Among other agents and conditions tested, imidazole increases Rh123 uptake in murine erythroleukemia cells (Tsiftsoglou et al 1983), *cis*-unsaturated fatty acids and cyclosporin reduce Rh123 uptake in lymphocytes

(Arslan et al 1984; Koponen et al 1985), hyperoxia reduces Rh123 uptake in microvascular cells (D'Amore & Sweet 1987), and injury induced by the phototoxicity of doxycycling, a photosensitive analogue of an antibiotic tetracycline, alters the morphology of mitochondria stained with Rh123 (Shea et al 1986). Laser surgery results in cells unable to take up Rh123 (Primrose et al 1987). Upon invasion of host cells, *Toxoplasma gondii* reduces Rh123 uptake (Tanabe & Murakami 1984).

MITOCHONDRIAL MEMBRANE POTENTIAL IN CANCER CELLS

As already mentioned, mouse, rabbit, and human bladder epithelial cells have low mitochondrial membrane potentials, which lead to a low uptake and short retention of Rh123 (Johnson et al 1982; Summerhayes 1982; Nadakavukaren et al 1985). In contrast, dimethylbenz(a)anthracene (DMBA)- or butyl nitrosamine (BBN)-transformed mouse bladder epithelial cell lines (MB48, MB49, BBN6), a benzo(a)pyrene-transformed rabbit bladder epithelial cell line (RBC), and human bladder carcinoma-derived cell lines (EJ, RT4, RT112) have significantly higher Rh123 uptake and retention (Summerhayes et al 1982; Nadakavukaren et al 1985).

These results have encouraged further comparison of Rh123 uptake and retention between normal epithelial cells and carcinoma-derived cells. The results of a six-year systematic study, overwhelmingly indicate that all normal epithelial cells tested have low mitochondrial membrane potential, hence, low Rh123 uptake and retention. In contrast, screenings of 200 cell lines/types derived from tumors of kidney, ovary, pancreas, lung, adrenal cortex, skin, breast, prostate, cervix, vulva, colon, liver, testis, esophagus, trachea, and tongue show that a great majority of adenocarcinoma, transitional cell carcinoma, squamous cell carcinoma, and melanoma have high Rh123 uptake and retention (Summerhayes et al 1982; Lampidis et al 1983; Wiseman et al 1985). The difference in mitochondrial membrane potential between normal epithelial cells and carcinoma cells is at least 60 mV (Modica-Napolitano & Aprille 1987; Davis et al 1985). The origin and consequence of this difference are still unknown. The most significant exceptions have been human oat cell and large cell carcinomas of lung, and poorly differentiated carcinoma of the colon (Summerhayes et al 1982; Chen et al 1985a). High Rh123 uptake and retention have not been detected in leukemias, lymphomas, neuroblastomas, or osteosarcomas (Summerhayes et al 1982; Chen et al 1983; Lampidis et al 1982; Nadakavukaren et al 1985). Intriguingly, Davis et al (1985) reported that in addition to mitochondrial membrane potential, plasma membrane potential is also higher in carcinoma cells than in normal epithelial cells.

Two poorly differentiated human colon carcinoma cell lines, FET and CCL 237, have extremely low mitochondrial membrane potentials that fail to increase in response to nigericin treatment (Modica-Napolitano & Chen 1988). These cells appear to have a low rate of respiration and to rely on glycolysis as the source of ATP, as postulated by Warburg (1956) for tumor mitochondria. However, these are the only two cases out of more than 200 tumor cell lines examined (own unpublished results) that fit the postulate. As expected, the growth of these carcinoma cells is exquisitely sensitive to the inhibition by 2-deoxyglucose, which normally has a low toxicity to most mammalian cells (Modica-Napolitano & Chen 1988).

Recently, v-fos oncogene-transformed fibroblasts have also been shown to have higher Rh123 uptake and retention than their untransformed counterparts (Zarbl et al 1987). In contrast, v-fes oncogene-transformed mink fibroblasts change the expression of their mitochondrial proton gradient from a state of high membrane potential and low pH gradient to a state of very low membrane potential and very high pH gradient (approximately 3-unit, Johnson et al 1982; Chen et al 1988). Other oncogenes, including src, ras, myc, fgr, mos, raf, sis, trk, EIA, and middle T (Zarbl et al 1987; own unpublished results), do not seem to affect the mitochondrial membrane potential.

EXPLOITATION OF MITOCHONDRIAL MEMBRANE POTENTIAL

As a Marker for Localization

Visualization of mitochondria by light microscopy dates back to 1857 when Kolliker first described filamentous and granular structures in muscle cells, which in hindsight must be mitochondria. Staining of these structures for detection was achieved by, among others, von Brunn, Altmann, Bensley and Benda who named the unit structure mitochondrion. One of the fruitful results from this era was the discovery of Janus green as a specific dye for mitochondria (Michaelis 1900). Intriguingly, Janus green is a lipophilic cation. Although quite toxic, it was used successfully by Lewis & Lewis (1915) to describe the behavior of mitochondria in living cells. Their findings were documented by hand-drawings made with remarkable precision. Among numerous dyes tested for mitochondrial staining at the turn of century, some were fluorescent dyes, most notably safranine, also a lipophilic cation (Colonna et al 1973; Akerman & Wikstrom 1976). After the development of fluorescence microscopy, more fluorescent dyes were tested. One of them, rhodamine 3B, a lipophilic cation, was used by

Johannes (1941) who, also by hand drawing, described the staining of mitochondria in living cells. Still others, 2-[4-(dimethylamino)styryl]-1-methylpyridinium and related analogs, were used to probe mitochondria in living cells (Bereiter-Hahn 1976, 1978; Bereiter-Hahn & Voth 1983; Bereiter-Hahn et al 1983). These compounds are, again, lipophilic cations. Hence, although the mechanism of specific uptake of Janus green, safranine, rhodamine 3B, or 2-[4-(dimethylamino)styryl]-1-methylpyridinium by mitochondria has not been resolved, all these compounds are lipophilic reagents with a delocalized positive charge. It is likely that all these lipophilic cations are taken up by mitochondria based on the lipid partitioning discussed above, the membrane potential–driven electrophoresis, and the Nernst equilibrium (Johnson et al 1981; Emaus et al 1986; Woolley et al 1987).

Using Rh123 at 10 μg/ml for 10 min, one might conclude that all the structures that take up the dye in living cells are mitochondria since a high membrane potential is a unique characteristic of mitochondria, and since Rh123 uptake is the result of this phenotype. This staining response could be usefully exploited for distribution studies since mitochondria in many living cell types still await localization (Table 1).

Rh123 has also been used to mark mitochondria when moved from one cell type to another (Clark & Shay 1982a,b), to follow mitochondria during subcellular fractionation (Casey & Anderson 1982), to monitor recovery from uncoupler FCCP treatment (Maro et al 1982), to compare species differences in dye uptake (Gupta & Dudani 1987) and to assess viability of *Mycobacterium leprae* in slit-skin smears (Odinsen et al 1986).

By marking mitochondria in living cells, Rh123 is also useful for following mitochondrial motility and morphological changes. 12-O-Tetradecanoyl-phorbol-13-acetate, a phorbol ester that activates protein kinase C, reversibly converts filamentous forms of mitochondria into granular forms (Chen et al 1984). It is possible that substrates for protein kinase C might be involved in mitochondrial morphology.

Intriguingly, certain mitochondrial outer membrane proteins have been suggested as potential targets for protein kinase C (Backer et al 1986). Salmeen et al (1985) reported that the predominant translational motion of mitochondria stained with Rh123 appeared to be Brownian in nature but occasionally superimposed on a slow uniform drift. The apparent diffusion coefficient is 5×10^{-12} cm^2 per second, and the drift speed is about 2×10^{-3} μm per second. It should be pointed out that these studies were made within a time span too short to detect the type of displacements made by mitochondria, first noted by Lewis & Lewis (1915). Nonetheless, the finding that mitochondria could be in a state of Brownian motion at certain times is intriguing since mitochondria appear to interact intimately with the cytoskeleton.

Table 1 Localization of mitochondria with rhodamine 123 by fluorescent microscopy

Cell types	References
Normal cells	
Sperms	Johnson et al 1980; Ahuja & Gilburt 1985; Robinson et al 1986
Sertoli cells	Tanphaichtr et al 1984; Robinson et al 1986
Oocytes	Albertini 1984; Herman & Albertini 1984
Fertilized eggs	Gundersen et al 1982
Preimplanted embryo cells	Batten et al 1987
Lymphocytes	Darzynkiewicz et al 1981; Arslan et al 1984; James & Bohman 1981
Granulocytes	Collins & Foster 1983
Macrophages	Johnson et al 1980; Koponen & Loor 1983
Cardiac muscle cells	Johnson et al 1980; Lampidis et al 1984
Myoblasts and myotubes	Summerhayes et al 1982; Chen et al 1982
Chondrocytes	Benel et al 1986; Champagne et al 1987
Fibroblasts	Johnson et al 1980; Goldstein & Korczack 1981
Microvascular endothelial cells	D'Amore & Sweet 1987
Adipocytes	DeMartinis et al 1988
Glomerular epithelial cells	Oberley et al 1982
Bladder epithelial cells	Wheeler & Arruda 1987
Salivary gland epithelial cells	Curtis et al 1986
Presynaptic nerve terminals	Yoshikami & Okun 1984
Altered cells	
Breast carcinoma cells	Summerhayes et al 1982
Colon carinoma cells	Chen et al 1985b
Lung carcinoma cells	Chen et al 1985b
Pancreas carcinoma cells	Summerhayes et al 1982
Adrenal cortex carcinoma cells	Hedberg & Chen 1986
Bladder carcinoma cells	Summerhayes et al 1982; Nadakavukaren et al 1985; Chen et al 1985b
Prostate carcinoma cells	Chen et al 1985b
Cervix carcinoma cells	Nadakavukaren et al 1985
Rous sarcoma virus-transformed cells	Johnson et al 1980
Feline sarcoma virus-transformed cells	Johnson et al 1982
Reovirus-infected cells	Sharpe et al 1982
Interferon-treated cells	Brouty-Boye et al 1981
Multidrug-resistant cells	Neyfakh 1988
Senescent cells	Martinez et al 1986
Parasites and their infected cells	
Plasmodium yoelii-infected mouse erythrocytes	Tanabe 1983; Izumo & Tanabe 1986; Izumo et al 1987
Toxoplasma gondii-infected mouse fibroblasts	Tanabe & Murakami 1984; Tanabe 1985
Plasmodium falciparum	Dibo et al 1985a,b; Geary et al 1986
Trypanosoma cruzi	Wolfson et al 1987
Plant cells	Wu 1987
Chlamydomonas reinhardii	Morris et al 1985
Yeast	Morris et al 1983; McCarthy et al 1987
Paramecium tetraurelia	Momayezi et al 1986

Associations between the mitochondria and the microtubules have previously been demonstrated (Raine et al 1971; Smith et al 1975, 1977; Wang & Goldman 1978; Heggeness et al 1978; Ball & Singer 1981, 1982). Using Rh123, Summerhayes et al (1983) confirmed the close distributional correlations between mitochondria and microtubules.

Intermediate filaments may also be involved in mitochondrial distribution. Earlier reports were consistent with this contention (Starger & Goldman 1977; Wang & Goldman 1978; Lee et al 1979; Toh et al 1980). However, in Rous sarcoma virus-transformed cells or cycloheximide-treated cells, the mitochondrial distribution correlates with the microtubule distribution but not with that of intermediate filaments (Ball & Singer 1982). Furthermore, when intermediate filament distribution was disrupted by microinjection of monoclonal antibodies recognizing a 95-kDa protein, neither motility nor distribution of mitochondria was affected (Lin & Feramisco 1981; Chen et al 1982; Summerhayes et al 1983). The relationship between the mitochondria and the intermediate filaments was further studied using Rh123. In many poorly differentiated human carcinoma cell lines, mitochondria cluster near the nucleus, leaving a large space in the cytoplasm devoid of mitochondria (Chen et al 1984). In these cells intermediate filament distributions, but not microtubule distribution, correlate closely with mitochondrial distribution (Chen et al 1984). In some but not all cell types (Mose-Larsen et al 1982), when microtubules are disrupted by colchicine or vinblastine, the distribution of coalesced intermediate filaments correlates with that of mitochondria (Summerhayes et al 1983), which is consistent with the results of previous studies (Starger & Goldman 1977; Destree & Hynes 1977). In CV-1 cells, cycloheximide disrupts the organization of intermediate filaments and the distribution of mitochondria, but not of microtubules (Summerhayes et al 1983; Sharpe et al 1980). In reovirus-infected cells, where the distribution of microtubules is similar to that found in uninfected cells, a concommitant disruption in distribution of mitochondria and intermediate filaments is observed (Sharpe et al 1982). It appears that both microtubules and intermediate

filaments can influence mitochondrial motility and distribution, but neither plays an absolute role. Other unknown factors and Brownian motion may also contribute to the final distribution of mitochondria. Organelles including mitochondria probably interact with the cytoskeleton in a complicated manner that awaits molecular elucidation (Mose-Larsen et al 1982).

As a Marker for Flow Cytometry

Rh123-stained cells are ideal for analysis of mitochondria concentrations and activities by flow cytometry (Shapiro 1985; Ronot et al 1986; Ratinaud & Julien 1987). Cells with varying mitochondrial number or mitochondrial membrane potential may be separated by a fluorescence-activated cell sorter. This method has been used to study various aspects of mitochondria in living cells (Table 2).

As a Means for Accumulating Drugs

Since the difference in mitochondrial membrane potential between carcinoma cells and normal epithelial cells is quite large, selective killing of carcinoma cells by lipophilic cations based on differential uptake and retention may be possible. Unexpectedly, this can be demonstrated by

Table 2 Use of Rhodamine 123 in flow cytometry

Aspects of mitochondria	References
Monitoring cybrids	Walker & Shay 1981, 1983; Clark & Shay 1982a,b; Hightower et al 1981; Kliot-Fields et al 1983
Lymphocyte activation and cell cycle	Darzynkiewicz et al 1981, 1982; James & Bohman 1981; Evenson et al 1985
Separation of bone marrow cells	Mulder & Visser 1987
Homing of bone marrow stem cells	Bertoncello et al 1985
Differentiation of HL-60 cells	Collins & Foster 1983
Differentiation of Friend cells	Tsiftsoglou et al 1983
Tumor heterogeneity	Sonka et al 1983
Effect of v-fes oncogene	Johnson et al 1982
Effect of v-fos oncogene	Zarbl et al 1987
Effect of anticancer drugs	Bernal et al 1982b; Adams et al 1984; Verhoef et al 1986
Drug-resistance	Sonka et al 1985
Effect of aging	Goldstein & Korczack 1981; Martinez et al 1986, 1987; Staiano-Coico et al 1982
Viability and motility of sperms	Evenson et al 1982
Effect of culture chondrocytes	Benel et al 1986; Champagne et al 1987
Characterization of liver cells	Doolittle et al 1987
Mitochondrial content	Steinkamp & Hiebert 1982

prolonged treatment (> 24 hr) of cells with Rh123, as opposed to 10 μg/ml for 10 min, the supravital condition described above (Lampidis et al 1983). Clonogenic assay with continuous exposure of cells to Rh123 for up to two weeks showed a selective inhibition of colony-forming ability in carcinoma cells but not in normal epithelial cells (Bernal et al 1982a). The basis for this selective toxicity during prolonged exposure to Rh123 is not completely understood. Darzynkiewicz's group reported that upon prolonged treatment, Rh123 cells are arrested at the G_1 phase of the cell cycle and gross morphological changes are found in mitochondria (Evenson et al 1985). In energized, tightly coupled mitochondria in vitro, Rh123 at 5 μg/ml inhibits ATP synthesis by 90% (Modica-Napolitano et al 1984; Mai & Allison 1983; Emaus et al 1984; Lampidis et al 1984; Benz et al 1987); uncoupler-stimulated F_0F_1ATPase activity is also inhibited. By using freeze-thawed mitochondria, in which segments of the respiratory chain may be directly assayed, Rh123 was found to have no effect on cytochrome *c* oxidase or succinate-cytochrome *c* reductase activities up to 40 μg/ml (Modica-Napolitano & Aprille 1987). This eliminates complexes II, III, and IV as possible targets. Because adenine nucleotide translocation in energized mitochondria is not affected, the above results suggest that F_0F_1ATPase could be the primary target. Indeed, direct inhibition of the activity of oligomycin-sensitive F_0F_1ATPase reconstituted in phospholipid vesicles by Rh123 has been demonstrated (Mai & Allison 1983). In addition, Rh123 was shown to inhibit mitochondrial protein synthesis (Abou-Khalil et al 1986), the transport of carbamyl phosphate synthetase I, ornithine transcarbamylase, and transhydrogenase into mitochondria (Morita et al 1982; Lubin et al 1987). Moreover, it inhibits bacterial DNA polymerase and RNA polymerase (enzymes that share some similarities with those of mitochondria) (Lameh et al 1987), and the activities of protein kinase C (O'Brian & Weinstein 1987) and calmodulin (Hait 1987). These inhibitory activities may all contribute to the cytotoxicity observed when cells with high mitochondrial membrane potential are exposed to Rh123 for longer than 24 hr. The failure of Rh123 at 10 μg/ml for 10 min to induce cytotoxicity probably results from (*a*) Only a relatively small amount of dye, although sufficient for visualization and quantitation, reaches mitochondria over 10 min (Nadakavukaren et al 1985); (*b*) As soon as cells are placed in a dye-free medium, the concentration gradient forces the dye to be released into the medium; (*c*) A brief inhibition of the enzymes mentioned above may not be cytotoxic; (*d*) The inhibition of these enzymes by Rh123 is probably reversible.

Potential anticarcinoma activity of Rh123 was also demonstrated in vivo (Bernal et al 1983; Arcadi 1986; Herr et al 1988). Control mice implanted intraperitoneally with 5×10^6 Ehrlich ascites carcinoma cells

had a median survival of 19 days (range 18–22 days). Tumor-bearing mice treated with a nontoxic dose of Rh123 (20 mg/kg on days 1, 3, 5) had a median survival of 50 days, with Treated/Control (T/C) = 260%. Treatment with only 2-deoxyglucose, an inhibitor of glycolysis (0.5 gm/kg on day 1), did not prolong survival. However, combined treatments with 2-deoxyglucose (0.5 gm/kg on day 1) and Rh123 (20 mg/kg on day 1, 3, 5) markedly prolonged survival, with T/C = 420%. Approximately 40% of the mice were cured (no evidence of tumor after 90 days). Rh123 has also been shown to be a sensitizer for the killing of tumor cells by hyperthermia (Kim et al 1985; Goffney et al 1986) and by photodynamic therapy (Powers et al 1986; Beckman et al 1987; Banes et al 1986; Castro et al 1987). Other lipophilic cations that accumulate in mitochondria and are useful for photodynamic therapy of tumor cells have also been reported; they include *N,N'*-bis(2-ethyl-1,3-dioxylene)kryptocyanine (Oseroff et al 1986; Ara et al 1987), pyronine Y and *o*-toluidine blue (Darzynkiewicz & Carter 1988), chalcogenapyrylium (Detty 1987; Powers 1987), and benzo(a)phenoxazinium (Foley et al 1987).

Based on these findings, further exploitation of the mitochondrial membrane potential for carcinoma therapy can be considered. The Nernst equation shows that if the plasma membrane potential is 60 mV and mitochondrial membrane potential 180 mV, the concentration of lipophilic cations inside mitochondria could theoretically be 10,000-fold greater than in the medium at equilibrium (Weiss & Chen 1984). Mitochondria occupy a significant portion of cell volume; thus, the total amount of lipophilic cations accumulated by mitochondria could be significant. Once the drug concentrations outside the cells drop, the drug inside mitochondria would, because of the concentration gradient, gradually be released into the cytoplasm and the extracellular medium. While the concentration gradient favors the release, the membrane potentials favor the retention of lipophilic cations inside the cells. The result of this competition might be that the lipophilic cations are slowly released into the cytoplasm. Thus, mitochondria could first serve as an accumulating reservoir, and then as an intracellular slow-releasing device for lipophilic cations. It should be possible to design such compounds that exert regulatory or cytotoxic effects outside the mitochondria. This offers a powerful new tool to target and kill undesirable cells, or to counteract infection by viruses. Mitochondria have never before been perceived and exploited as a depot and slow-releasing device for drugs. But an example of the potential of this approach is described below.

Dequalinium has been used for about thirty years as an antimicrobial agent in topical ointments, paints, and in over-the-counter sore throat lozenges and mouthwash. It is a lipophilic cation with two delocalized

positive charges. It is accumulated by mitochondria (Weiss et al 1987), where it can inhibit mitochondrial electron transport (Anderson et al 1988). Upon release from mitochondria it can intercalate into DNA (Wright et al 1980), and it can also inhibit the activities of calmodulin and protein kinase C (Hait 1987). When cells are exposed to dequalinium for 3 hr, it is 125-fold more toxic to human carcinoma cells such as MCF-7 than to normal epithelial cells such as CV-1 (Weiss et al 1987). Dequalinium was effective in vivo against MB49, a DMBA-transformed mouse bladder carcinoma implanted intraperitoneally, in 25 experiments with T/C = 250% (an average of 190%) (Weiss et al 1987). Dequalinium is also effective against subcutaneously implanted tumors including MB49, CX-1 (human colon carcinoma in nude mice), and W163 (1,2-dimethylhydrazine-induced rat colon carcinoma). It inhibits tumor growth by about 50% (Weiss et al 1987). The most significant activity is observed against mammary tumors induced in situ by the carcinogen DMBA (Chen et al 1985b). In this model, which avoids transplantion, dequalinium inhibits growth and leads to regression of tumors. Dequalinium also prolongs the survival of nude mice bearing human ovarian carcinoma up to T/C = 360% (N. Teng, personal communication).

Lipophilic cations may also be used as a carrier for other drugs that have no positive charge and whose uptake by tumor cells is not driven by the mitochondrial membrane potential. A complex between two molecules of Rh123 and one molecule of cisplatinum has been made (Teicher et al 1987). The complex is taken up by tumor cells in response to membrane potential; thus, the accumulation is greater in carcinoms cells than in normal cells (Teicher et al 1986; Abrams et al 1986). Since platinum is a radiosensitizer, the tumor cells also become more sensitive to radiation (Teicher et al 1987).

CONCLUDING REMARKS

It is very difficult to explain the phenomenon of specific and extraordinarily high uptake of Rh123 by mitochondria in living cells by a mechanism other than a high mitochondrial membrane potential, which has repeatedly been questioned by Tedeschi (1980). Why is the closely related, but neutral analog rhodamine 110 not taken up by mitochondria? Why is the delocalized positive charge essential? How does one explain the effect of ionophores and mitochondrial inhibitors, in particular, nigericin, which dramatically increases the uptake in certain cells? How does one explain the correlation between Rh123 uptake and membrane potential artificially set up by valinomycin and K^+ in isolated mitochondria?

In hindsight, it was fortunate that Rh123, instead of rhodamine 3B, 6G,

or other rhodamine analogs, was chosen in the initial experiments. Had other rhodamines been used, the phenomenon might be explained by lipid partitioning since the combination of azide and oligomycin would not prevent the uptake of rhodamines other than Rh123 by mitochondria in living cells. Under fluorescence microscopy, it is difficult for the human eye to distinguish the uptake due to lipid partitioning from that due to the membrane potential. Hence, the involvement of the latter could be mistakenly excluded. However, these findings also suggest that membrane potential–independent uptake of lipophilic cations other than Rh123 deserves further investigation. Why don't these lipophilic cations stain the plasma membrane, the Golgi apparatus, or the nuclear envelope? Are specific lipids known to be enriched in mitochondria responsible, at least in part, for the uptake and retention of these other lipophilic cations?

Based on the results reviewed here, it appears that a significant proton motive force does exist across the mitochondrial inner membrane in living cells. However, not all cell types have mitochondria with equally large proton gradients. The bioenergetic states of mitochondria in different cell types of the same species could be quite different. Cardiac muscle cells seem to have the most active mitochondria, whereas bladder epithelial cells and resting lymphocytes have the lowest. Conceivably, some investigators might have used cell types with inherently low proton gradients in their experiments to argue against Mitchell's theory. Moreover, the expression of the proton gradient as pH gradient or membrane potential may vary among different cell types. Cardiac muscle cells appear to express it almost completely as membrane potential, whereas many cell types express it both as membrane potential and pH gradient. The basis for these variations and their regulations is completely unknown. Intriguingly, when cells with different mitochondrial membrane potentials are fused, mitochondria adopt the same level of potential only when the cytoskeleton elements of the two cells are fully intermixed.

Despite the 50-year old debate, the question whether mitochondria in tumor cells differ from those in normal cells remains unresolved. Observations made with Rh123 show that oncogenic transformation does affect mitochondria but not in a universal way. Oncogene v-fos leads to hyperpolarization of mitochondria, but oncogene v-fes yields the opposite result. Differentiated carcinoma cells have high mitochondrial membrane potentials, but poorly differentiated carcinoma cells and oat cell carcinoma do not. In addition, oncogenesis could affect mitochondrial morphology, distribution, size, and number (Chen et al 1984, 1985b; Pedersen 1978). However, nothing is known about the consequence of these changes or their origin. Hopefully, further studies on v-fos and v-fes oncogenes will shed light on these questions in the near future.

Fruitful results with Rh123 have encouraged studies on other organelles in living cells with fluorescent lipophilic cations in conjunction with confocal scanning laser microscopy (White et al 1987). The endoplasmic reticulum has been successfully localized in living cells with 3,3′-dihexyloxacarbocyanine (Terasaki et al 1984, 1986; Lee & Chen 1988). The Golgi apparatus and nuclear envelope are attractive future targets for visualization and localization in living cells.

Potentially the most significant discovery resulting from the use of Rh123 is that there is a new way to bring compounds into cells and mitochondria. By exploiting the membrane potential, delocalized cations may be moved across lipid bilayers electrophoretically. A 60 mV potential across 5-nm plasma membrane is equivalent to 120,000 V across 1 cm. Numerous drugs that have a low permeability or depend on transport systems could take advantage of this electric field for facilitating entry into cells.

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chemotherapy

<pharmacology, oncology> The treatment of disease by means of chemicals that have a specific toxic effect upon the disease producing microorganisms (antibiotics) or that selectively destroy cancerous tissue (anticancer therapy).

(12 May 1997)

Previous: chemotherapeutic, chemotherapeutic agent, chemotherapeutic index, chemotherapeutics
Next: chemotherapy, adjuvant, chemotherapy drug sensitivity test, chemotic

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